Attorney's Docket No.: 06275-277001 / AFG/Z70389-1P US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : John Edward Norris Morten
Serial No. : 09/787,371
Filed : March 16, 2001
Title : POLYMORPHISMS IN THE HUMAN VCAM-1 GENE, SUITABLE FOR DIAGNOSIS AND TREATMENT OF VCAM-1 LIGAND MEDIATED DISEASES

Art Unit : 1634
Examiner : Carla J. Myers

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. §1.132 OF DR. JOHN E. N. MORTEN

I, John E. N. Morten, a citizen of the United Kingdom, residing in Northwich, England hereby declare as follows:

1. I have been a research scientist at AstraZeneca UK Ltd. (formerly Zeneca Ltd.) in the United Kingdom since 1987. I am currently a Team Leader in the Global Enabling Science and Technology Department at AstraZeneca. From 1977 to 1980, I was a postgraduate researcher at University of Birmingham, UK. I received my Ph.D. in Cancer Studies from University of Birmingham in 1980. I have conducted molecular and biochemistry research for 20 years.

2. I am the inventor of the invention claimed in the above-identified patent application, and I have read and understand the contents of the present patent application. This application claims priority to PCT/GB99/03057, filed September 15, 1999, and GB 9820338.3, filed September 19, 1998.

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the ~~Commissioner for Patents, Washington, D.C. 20231~~. US Patent & Trademark Office, Arlington, VA

January 22, 2003
Date of Deposit

Lisa Gray
Signature

Lisa Gray
Typed or Printed Name of Person Signing Certificate

3. I have been advised and understand that the Examiner has rejected claims 1-4, 6 and 7 of the above-referenced application for being indefinite over the recitation of "EMBL ACCESSION NO: M92431." The Examiner argues that the sequence represented by that accession number may not currently be identical to what was represented by that accession number at the priority date.

4. Attached hereto as Exhibit A is a paper copy of the sequence of EMBL ACCESSION NO:M92431. I hereby declare that the sequence represented by Exhibit A is identical to the sequence of EMBL ACCESSION NO:M92431 that existed at the time the earliest priority application was filed (September 19, 1998).

5. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

John E. N. Morten
John E. N. Morten

7th January 2003
Date

declaration.doc

EXHIBIT A

M92431

aattcgataattcaaaatgatatttcagtggggacaaggccaaaccatattatgtgc
tttatgctactaataaaaaggttaaaccgaacaagttcaaagacaaaactcagtagt
actttattcagacaggttagtctaaatctgttaaccttatacttgcaactctgatca
ttcattaattctgcaaattttaataaatgctttattttaagctaaatgctgagatga
aaaaatgaaaccatatgagttagcaaagtagaaaatataggcatattaatcagtaaa
tgcagaatgataaatgctccatcaatatgcacttgttgtagtgaggccaccgaggag
ggtgcaatcctctcaacctgggaggagcaggtaggacttcagatgtcatccaactca
aagatatagtgagggacttgatcaaacatttgccaagaccactatgagttaaatgaa
tagattaggcatttctccaatgttgcaagcttcgaatcatatccaaactcagaacaa
catagcttggtcataatgatcccaaggatcctattggccattgtctttgagcctcaa
aggaacatattaaaactccataataccctttgatctattctgaagttaagtagtga
atttacatgatgatgacacaaacactgtaaaggacctctgggttacttgtttataag
ctagtatttcctgaatcaatttttctgatccctagatatttggtaggtgaagtcata
cctatatatccccacaccctagaacagcatctccaacttttttttccctccttgtct
tttagtgggagccacatcagtatccaaggaggagatccagaagcctctccaaccagg
tagggacagttatagattccagacctcagctatggcctttgttacagagtacaaatg
ttatatagtacaagtttattgtacacatcccattgagtctctgagctttagaatttt
cttgtagaatttaacagtttttttcatgccgtatttacatattattgctagtatttag
aattttcttctccaaatgtataacgtttattattgcatttttttgtatccactaagtg
gaaaatcatgcattagatattgtagaagtagatacaacaatgaacaagaactggtcc
tgaccatgagaggaactgatgatccaatgggggagatagacctgcacgtgtttaata
aaaggaagtggctattccggtttctttttgatgggcaagcattttgcaaggccttgg
gctatgtgtgtgcaaggctaagccagttagttaattgggatttttttaaaaaggcac
ttcactgggggggaaaaggaacatagagttggttattgtccccttgcctataataaaa
acctattatttttaatttttttaactgggtttgcggttaaatctcacagcccaagaga
tttgccacttcagatggattccatacacttgcatttaagtatgcaaaaaaattccaa
ttatccagcaatttaaccaaattattggtaacttttctaaaacaaaaaaaaattgtt
tcccttgttttggcagcaatttcagttacagtcctttacttttctactcaagaaaata
gtttcaaaaagttgatgtttgttgctaaaagaactatttttatgaataaatataaaa
ctaagaagttatggtgtccctttttttaaaaaatgactcatcaaaagaaataacttttt
tcctttctcttgtaagagaaaaaaattaatctcttttagaattgcaaacatatttcc
ttgatggagaaaatcaattcacatggcatagtcgttatttatccagttcaaaaacca
gagtagaatttactactctgtctccatttttttctctccccacccccttaacccacat
tggattcagaaagcttcattctgcaatcagcattgtcctttatctttccagtaaaga
tagccttttggagtcgaagatgaggaaaagcctgtattttatagtcttggaagtgtc
ttcttttgccaggacagagagaggagcttcagcagtgagagcaactgaaggggttaa
tagtggaacttggctgggtgtctgttaaacttttttccctggctctgccctgggttt
ccccttgaagggatttccctccgcctctgcaacaagacccttttataaagcacagact
ttctatttcactccgcggtatctgcatcgggcctcactggcttcaggagctgaatac
cctcccaggcacacacaggtgggacacaaataagggttttggaaccactattttctc
atcacgacagcaacttaaaatgcctgggaagatggtcgtgatccttggagcctcaaa
tatactttggataatgtttgcagcttgtaagttatttcccttcatctgtttcaaatg
tt

Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3943–3947, May 1993
Biochemistry

Vascular cell adhesion molecule 1: Contrasting transcriptional control mechanisms in muscle and endothelium

(tissue specificity/cytokines/promoter activity)

MICHAEL F. IADEMARCO, JAY J. MCQUILLAN, AND DOUGLAS C. DEAN*

Departments of Medicine and Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110

Communicated by Philip W. Majerus, December 31, 1992 (received for review September 17, 1992)

ABSTRACT **Interaction between vascular cell adhesion molecule 1 (VCAM-1), which appears on the surface of endothelial cells in response to inflammation, and its integrin counter receptor, $\alpha 4\beta 1$, on immune cells is responsible for targeting these immune cells to cytokine-stimulated endothelium. In addition to its role in the immune system, VCAM-1 is also expressed in a developmentally specific pattern on differentiating skeletal muscle, where it mediates cell–cell interactions important for myogenesis through interaction with $\alpha 4\beta 1$. In contrast to endothelium, there is high basal expression of VCAM-1 in skeletal muscle cells and the expression is not cytokine-responsive. Here, we examine the molecular basis for these contrasting patterns of expression in muscle and endothelium, using VCAM-1 promoter constructs in a series of transfection assays. In endothelial cells, octamer binding sites act as silencers that prevent VCAM-1 expression in unstimulated cells. Tumor necrosis factor α overcomes the negative effects of these octamers and activates the promoter through two adjacent NF-κB binding sites. In muscle cells, a position-specific enhancer located between bp −21 and −5 overrides the effect of other promoter elements, resulting in constitutive VCAM-1 expression. A nuclear protein binds the position-specific enhancer in muscle but not endothelial cells; thus the pattern of expression of this protein could control enhancer activity.**

Vascular cell adhesion molecule 1 (VCAM-1) is a member of the immunoglobulin gene superfamily that is expressed on the surface of endothelial cells in response to inflammation (1–3). Through its interaction with the integrin receptor $\alpha 4\beta 1$ (4)—and also perhaps $\alpha 4\beta 7$ (5)—on T cells, monocytes, and eosinophils, VCAM-1 targets these immune cells to cytokine-stimulated endothelium (1, 6–12). VCAM-1 and $\alpha 4\beta 1$ mediate cell–cell interactions which are important for skeletal muscle differentiation (13).

In contrast to endothelial cells, where VCAM-1 expression is dependent upon cytokines (14), we show that there is high basal expression of VCAM-1 on muscle cells and that this expression is not affected by cytokines. To investigate the basis for these contrasting patterns of expression, the activity of VCAM-1 gene promoter constructs was examined in the two cell types. In endothelial cells, "octamer-like" motifs in the promoter act as silencers to prevent VCAM-1 expression in unstimulated cells, and tumor necrosis factor α (TNF) overcomes the negative effects of these octamers by activating the promoter through two adjacent NF-κB binding sites. However, in muscle cells, an element 3′ of the TATA box overrides the activity of other promoter elements, resulting in constitutive VCAM-1 expression. This element is position-dependent and we present evidence that its activity is controlled by the distribution of its nuclear binding protein.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. Human umbilical vein endothelial cells (HUVECs) (14), C2C12 mouse myoblasts (15), Raji Burkitt lymphoma cells (16), and P19 embryonic carcinoma cells (17) (differentiated with retinoic acid) were grown as described.

Approximately 1–5 × 10^6 cells were transfected by a calcium phosphate technique (14) with 18 μg of reporter plasmid and 2 μg of pRSV/L, which contains the Rous sarcoma virus long terminal repeat fused to the firefly luciferase gene. Luciferase assays were done as described (16). Raji cells were transfected by electroporation as described (16). Protein extracts were made 36 hr after transfection and chloramphenicol acetyltransferase (CAT) activity was determined (18). Approximately 20 μg of protein from HUVECs, 100 μg from C2C12 cells, 400 μg from Raji cells, and 200 μg from differentiated P19 cells were used for CAT assays and reactions were allowed to proceed for 3–5 hr. Acetylation of [^{14}C]chloramphenicol was quantified by thin-layer chromatography followed by scintillation counting.

Immunofluorescence. Immunofluorescent staining for VCAM-1 was done as described (13). VCAM-1 was detected on HUVECs with the monoclonal antibody BB5 (R & D Systems, Minneapolis) and on C2C12 cells with the monoclonal antibody M/K-1 (19).

RNA Analysis. Poly(A)$^+$ RNA was isolated by using the Micro-FastTrack system (Invitrogen, San Diego), and Northern blot analysis to detect VCAM-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs was done as described (13).

Plasmid Construction. VCAM-1 promoter constructs (14), the minimal promoter construct pTA-CAT (20), and the minimal promoter construct containing two VCAM-1 NF-κB sites, pTA(−77/−63)CAT (14), have been described. pTA(−77/−63)(−1554)CAT was constructed by cloning annealed oligonucleotides containing the octamer at bp −1554 (underlined) and the two NF-κB sites at bp −77 and −63 (bold), 5′-agctTAGTGAATTTACATGATGATGAagatct-TGCCCTGGGTTTCCCCTTGAAGGGATTTCCCTC-CGCCa-3′, into the *Hin*dIII site (terminal nucleotides shown in lowercase type were added to create *Hin*dIII sites).

pSVCAT (pCAT control, Promega) contains the simian virus 40 (SV40) early promoter and enhancer driving the CAT reporter gene. pSV(−1554)CAT was created by cloning annealed oligonucleotides containing the octamer at bp −1554, 5′-gatctGTAGTGAATTTACATGATGATGA-3′ (nucleotides in lowercase type were added to create *Bgl* II sites), into

Abbreviations: VCAM-1, vascular cell adhesion molecule 1; CAT, chloramphenicol acetyltransferase; TNF, tumor necrosis factor α; HUVEC, human umbilical vein endothelial cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SV40, simian virus 40; ATF, activating transcription factor.

*To whom reprint requests should be addressed at: Box 8052, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110.

the *Bgl* II site of pSVCAT. In pSV(−1554)CAT-M the G and TAC in italics are mutated to T and ATT, respectively.

pTA-ATF-CAT contains an activating-transcription-factor (ATF) binding site cloned into the minimal promoter construct pTA-CAT (20). pTA-ATF(−1554)CAT was created by cloning annealed oligonucleotides containing the octamer at bp −1554 (with *Hin*dIII sites on both ends) into the *Hin*dIII site of pTA-ATF-CAT. pTA-ATF(IgH)CAT was constructed by cloning the octamer binding site from the immunoglobulin heavy-chain gene [5′-agcttGAGAATATGCAAATCAATTGa-3′ (21); lowercase letters correspond to nucleotides added to facilitate cloning into a *Hin*dIII site] into the *Hin*dIII site of pTA-ATF-CAT.

pTA-FN-CAT contains fibronectin gene promoter sequences between bp −36 and +8 (20). Annealed oligonucleotides containing the position-specific enhancer [bp −23 (*Xba* I) to −1 (*Hin*dIII) of the VCAM-1 gene] were cloned into a multiple cloning site immediately upstream of the TATA box in pTA-FN-CAT.

Gel Retardation Assays. Nuclear protein extracts were prepared and gel retardation assays were done as described (14). The probe for the position-specific enhancer spanned bp −23 to +11 of the VCAM-1 gene. Probes for the octamers correspond to the double-stranded oligonucleotides used in plasmid construction described above. The ATF probe was described previously (22).

RESULTS

Contrasting Patterns of VCAM-1 Expression in Endothelial Cells and Muscle Cells. VCAM-1 expression in HUVECs is dependent upon cytokines such as TNF (Fig. 1*A* and ref. 14). However, there is high basal expression in the C2C12 myoblast cell line and TNF has no stimulatory effect (Fig. 1*B*). Previously, we found that VCAM-1 expression in endothelial cells is controlled at the level of transcription: silencers prevent expression in unstimulated cells, and TNF activates the promoter through NF-κB sites (14). In C2C12 cells, the level of VCAM-1 mRNA parallels that of VCAM-1 protein (Fig. 1*C*), indicating that expression of VCAM-1 in C2C12 cells could also be controlled at the level of transcription.

To examine the basis for these patterns of expression, we initiated a series of transfection assays with different VCAM-1 gene promoter constructs, using HUVECs as a model of endothelial cells and C2C12 cells as a model of skeletal muscle cells.

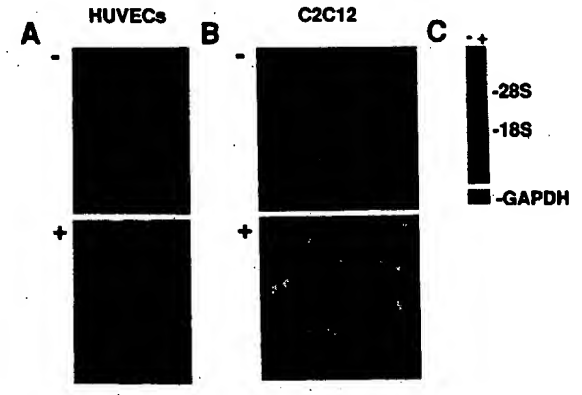

FIG. 1. VCAM-1 expression is dependent upon TNF in endothelial cells but is constitutive in muscle cells. Where indicated (+ and −), TNF was added at 10 ng/ml for 8 hr. (*A*) HUVECs were stained with the monoclonal antibody BB5 and visualized with fluorescent microscopy. (*B*) C2C12 cells were stained in the same fashion, but antibody M/K-1 was used to detect VCAM-1. (*C*) Northern blot of 5 μg of C2C12 RNA. Positions of 28S and 18S rRNA are shown. Hybridization with GAPDH cDNA (*Lower*) was used to control for the amount of RNA loaded.

Octamers Prevent Expression of VCAM-1 on Unstimulated Endothelial Cells. Previously, we found that the activity of VCAM-1 gene promoter constructs containing either 2.1 or 1.6 kb of VCAM-1 gene 5′ flanking sequence was not above background in unstimulated HUVECs (14). However, TNF activated the promoter, and this was mediated through two adjacent NF-κB sites at bp −63 and −77. Consecutive deletion in the VCAM-1 promoter from kb −1.6 to bp −933 and then to bp −288 caused a progressive increase in promoter activity, suggesting that silencers were being sequentially removed (Fig. 2 and ref. 14). Originally, we identified three octamers in this region (at bp −1154, −1180, and −733) (14); however, there are at least 10 other sites in this region that show similarity to octamers.

Binding of protein to the VCAM-1 gene octamer at bp −1554 was compared with binding to a known octamer from the immunoglobulin heavy-chain gene (IgH) (Fig. 3*A*). Both octamers showed two protein complexes, but the more rapidly migrating complex was generally less abundant with the IgH octamer. The IgH octamer competed effectively for binding to the VCAM-1 gene octamer. However, the VCAM-1 gene octamer was only partially effective in competing for binding to the IgH octamer, suggesting that the IgH octamer may be a higher-affinity binding site or may bind proteins in addition to those bound by the VCAM-1 gene octamer. Mutation of 4 bp in the −1554 octamer, which prevents function (Fig. 3*B*), eliminated its ability to compete for binding of protein to octamers (Fig. 3*A*).

We tested the effect of individual VCAM-1 gene octamers as silencers on heterologous promoters. Octamers at bp −1180 and −1554 inhibited the activity of both a simple promoter containing only a TATA box and an ATF site and a relatively complex promoter containing the SV40 early gene promoter/enhancer (Fig. 3 *B* and *C*). These results confirm that VCAM-1 octamers are silencers in HUVECs.

To determine whether this inhibitory effect of the VCAM-1 gene octamers also occurred with other octamers, we tested the effect of the well-characterized IgH octamer on promoter activity in HUVECs (Fig. 3*B*). The IgH octamer was also an efficient silencer, suggesting that HUVECs contain a com-

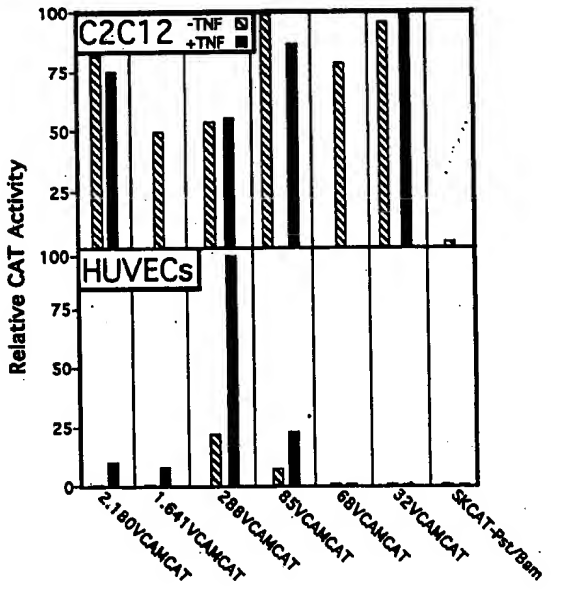

FIG. 2. Activity of VCAM-1 promoter constructs in HUVECs and C2C12 cells. Numbers in the name indicate the amount of VCAM-1 gene 5′ flanking region in each construct. Results are an average of duplicate samples from at least four separate experiments.

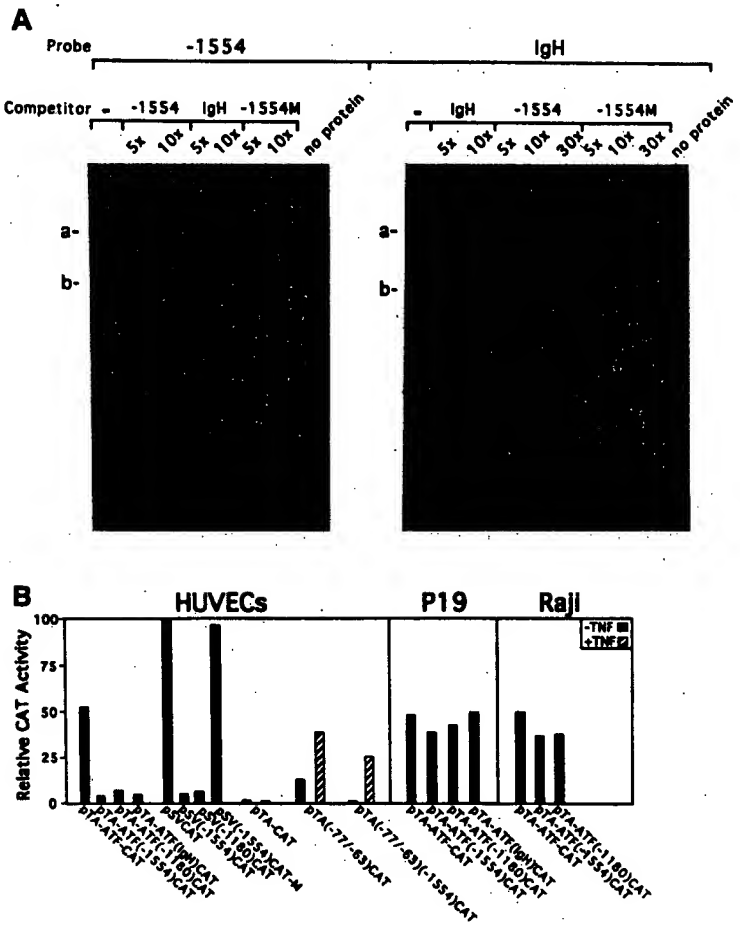

FIG. 3. Role of octamers in controlling VCAM-1 expression. (*A*) Gel retardation assay comparing binding of HUVEC proteins to the VCAM-1 gene octamer at bp −1554 and a control octamer from the immunoglobulin heavy-chain gene (IgH). Specific complexes (a and b) are indicated. Numbers indicate the molar excess of unlabeled competitor. −1554M is an inactive mutant octamer (see *B*). (*B*) Octamer activity is cell type-specific. pTA-ATF-CAT is a simple promoter construct containing a TATA box and an ATF site driving the CAT gene. Octamers at bp −1180 and −1554 and the IgH octamer were cloned upstream of the ATF site in this construct to create pTA-ATF(−1180)CAT, pTA-ATF(−1554)CAT, and pTA-ATF(IgH)CAT. Octamers at positions −1180 and −1554 were cloned into pSVCAT to create pSV(−1180)CAT and pSV(−1554)CAT. pSV(−1554)CAT-M contains the mutant −1554 octamer. The octamer at position −1554 was cloned into pTA(−77/−63)CAT, which contains the VCAM-1 gene NF-κB sites upstream of the SV40 early-gene minimal promoter, to create pTA(−77/−63)(−1554)CAT. Constructs were transfected into HUVECs, retinoic acid-differentiated P19 cells, and Raji cells.

plement of octamer-binding proteins that direct octamers to act as silencers.

The octamers were not silencers in the context of either the ATF or the SV40 promoter construct in the retinoic acid-differentiated embryonic carcinoma cell line P19 or in Raji B cells (Fig. 3*B*). Octamers have been shown previously to be transcriptional silencers in undifferentiated P19 cells, but this activity is lost upon differentiation (23). Our results then provide further evidence that the activity of octamers is cell type-specific.

We found that octamers did not prevent TNF-responsiveness of NF-κB sites. The octamer at bp −1554 was cloned upstream of the NF-κB sites in pTA(−77/−63)CAT to create pTA(−77/−63)(−1554)CAT, and the effect of the octamer was tested in transfection assays in HUVECs. Inclusion of the octamer did not prevent TNF responsiveness (Fig. 3*B*). However, in addition to conveying TNF responsiveness, the NF-κB sites also increased basal activity of the minimal promoter [compare the basal activity of pTA(−77/−63)CAT with that of the parent construct, pTA-CAT, in Fig. 3*B*]. The octamer decreased the basal activity to background. Treatment with TNF had no effect on the octamer activity, nor did it affect the interactions of octamers with proteins (data not shown). The combination of NF-κB sites and the octamer in pTA(−77/−63)(−1554)CAT seems to mimic the whole VCAM-1 promoter in endothelial cells: activity is not above background until cells are treated with TNF. We propose that the octamers serve to prevent VCAM-1 expression in unstimulated endothelial cells, whereas the NF-κB sites are responsible for activating the gene in response to inflammatory cytokines.

A Position-Specific Enhancer Causes Constitutive VCAM-1 Expression in Muscle Cells. In contrast to HUVECs, the VCAM-1 promoter had a high level of basal expression in C2C12 cells, and TNF had no effect on promoter activity (Fig. 2). A series of 5′ deletion mutants were transfected into C2C12 cells to determine what sequence was important for this pattern of expression. Surprisingly, deletion all the way to bp −32 had little effect on promoter activity, suggesting that an element 3′ of the TATA box is responsible.

The lack of TNF responsiveness in C2C12 cells suggested that VCAM-1 gene NF-κB sites might not be responsive to TNF in muscle cells or that the activating element was overriding the effect of the NF-κB sites. To distinguish

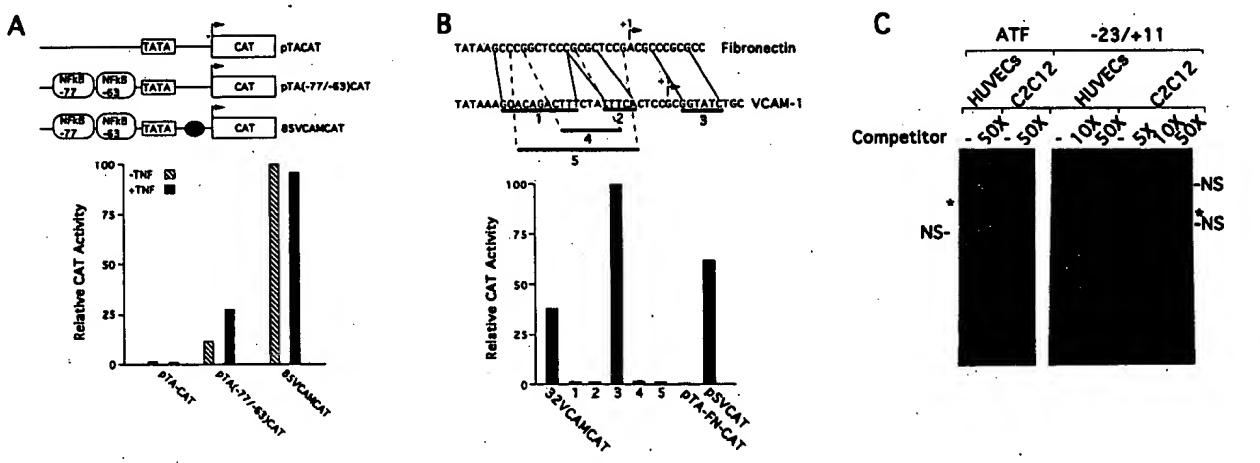

FIG. 4. An element 3′ of the TATA box is responsible for constitutive VCAM-1 expression in muscle. (*A*) Diagrams of reporter plasmids. The filled circle represents the position-specific enhancer. (*B*) Localization of the position-specific enhancer. To more precisely localize the enhancer, VCAM-1 gene promoter sequences between positions −23 and +11 were systematically replaced with corresponding regions of the human fibronectin gene promoter, which does not contain the enhancer (note that the activity of pTA-FN-CAT, a minimal fibronectin promoter construct containing the region between bp −36 and +8, is not above background). Lines indicate the extent of each mutation. The activity of the mutants was tested in transfection assays in C2C12 cells. (*C*) Binding of nuclear protein correlates with activity of the position-specific enhancer. Binding of nuclear protein to the VCAM-1 promoter between bp −23 and +11 (see *B*) was compared for extracts from HUVECs and C2C12 cells in a gel retardation assay (*Right*). As a control, nuclear extracts were compared for their ability to bind to an activating transcription factor (ATF) site (*Left*). Stars denote specific complexes, and "NS" denotes nonspecific complexes. "Competitor" indicates that unlabeled probe was included in the assay. Numbers give the molar excess of unlabeled probe.

between these possibilities, the activity of the NF-κB sites was compared in the presence or absence of the element. In the absence of the element, the NF-κB sites were responsive to TNF (Fig. 4*A*), suggesting that the element can override the activity of these sites.

Deletions that remove the octamers had little effect on promoter activity in C2C12 cells (Fig. 2*B*), implying either that the octamers are not negative elements in C2C12 cells or that the activating element also overrides the effect of the octamers. In the absence of this element we found that the octamers did act as silencers in C2C12 cells. Therefore, this activating element also overrides the effect of the octamers in C2C12 cells.

To localize the activating element, a series of mutations were made. Mutations were created by replacing VCAM-1 gene promoter sequences between positions −32 and +12 with corresponding sequences from the fibronectin gene promoter (24), which lacks the activating element (Fig. 4*B*).

Mutation of VCAM-1 gene promoter sequences between positions −21 and −5 inhibited promoter activity, whereas mutation of sequences between positions +2 and +11 did not (Fig. 4*B*), suggesting that sequences between positions −21 and −5 are necessary for activation. Three additional mutations within this region (positions −21 to −13, positions −14 to −8, and positions −9 to −5) also inhibited promoter activity (Fig. 4*B*), indicating that the element(s) required for activation overlaps these three mutations. The mutation between positions +2 and +11 reproducibly stimulated CAT activity, suggesting that it could be removing an inhibitory element. However, since this region is 3′ of the transcriptional start site (indicating that it is included in the VCAM-1-CAT mRNA), it is possible that the mutation increases mRNA stability. None of the mutations showed activity in HUVECs (data not shown).

The region between positions −23 and −1 was cloned immediately 5′ of the TATA box in pTA-FN-CAT. However, it had no effect on promoter activity in transfection assays into C2C12 cells (data not shown), suggesting that it is a position-specific enhancer whose location 3′ of the TATA box is necessary for function.

Binding of a Nuclear Protein Correlates with Activity of the Position-Specific Enhancer. Protein extracts from HUVECs and C2C12 cells were examined for binding to the position-specific enhancer. A specific complex was apparent with extracts from C2C12 cells, but not HUVECs (Fig. 4*C*). Mutations that inhibit function of the position-specific enhancer prevented binding of the nuclear protein (data not shown). As a control, HUVEC and C2C12 extracts showed similar levels of binding to an ATF site—ATFs are ubiquitous and we have found similar levels of binding activity in most cell lines. We conclude that the pattern of nuclear protein binding controls the activity of the position-specific enhancer.

DISCUSSION

We have examined mechanisms responsible for the contrasting patterns of VCAM-1 in endothelium and skeletal muscle (summarized in Table 1). Octamers prevent basal VCAM-1 gene promoter activity in endothelial cells. TNF overcomes the effect of these octamers and activates transcription through two adjacent NF-κB sites.

A number of transcription factors can bind to octamers and their activity has been found to vary in different cell types (25, 26). In support of such cell-type specificity, we found that octamers did not show inhibitory activity in B cells or in differentiated P19 cells. The transcriptional activity of octamers changes when P19 cells are stimulated with retinoic acid to differentiate along a neuronal pathway: in undiffer-

Table 1. Summary of VCAM-1 gene promoter element activity in endothelial and muscle cells

	Contribution of specific elements			Net promoter activity	
Cell type	Octamer	NF-κB sites (+ TNF)	Position-specific enhancer	− TNF	+ TNF
Endothelial	↓	↑↑	0	−	+
Muscle	↓	↑↑	↑↑↑	+	+

entiated cells they act as silencers, whereas this activity is lost upon differentiation (23). We have found that VCAM-1 gene expression is activated upon differentiation of P19 cells, and this results at least in part from the fact that VCAM-1 gene octamers lose their capacity to silence transcription as a result of differentiation (unpublished results). Thus, the role of octamers in the VCAM-1 gene promoter may extend beyond simply preventing VCAM-1 expression in unstimulated endothelial cells.

Octamers and NF-κB sites are functional in muscle cells, but an element 3′ of the TATA box—which is active in muscle but not in endothelial cells—overrides their activity, resulting in constitutive activation of the VCAM-1 promoter. Activity of this element correlates with binding of a nuclear protein, suggesting that the protein could control activity.

Not only is VCAM-1 expressed in contrasting patterns on endothelium and muscle, its expression on muscle is developmentally specific: it is present on differentiating skeletal muscle but not on adult muscle fibers (13). Additionally, we have found that VCAM-1 is expressed in a number of other developing tissues (unpublished results). Thus, it is possible that this element controls the developmental pattern of VCAM-1 expression.

The location of the position-specific enhancer between the TATA box and the transcriptional start site suggests that it could be an initiator element (Inr) which directs specific transcriptional initiation (27). In support of this possibility, the sequence from bp −6 to +2, 5′-CACTCCGC-3′, is similar to the Inr consensus, YAYTCYYY (Y is C or T), and a mutation over a portion of this region (bp −9 to −5) inhibited promoter activity. However, unlike Inr sequences, the VCAM-1 element controls tissue specificity. Furthermore, mutations that were clearly upstream of the Inr consensus (bp −23 to −13 and bp −14 to −8) were equally effective at blocking activity. This could indicate that an element immediately upstream of the Inr sequence is also important for activity. Conceivably, the combination of a second element with Inr could result in the tissue specificity. Alternatively, since the consensus sequence for Inr is not strict (see above), this region of the VCAM-1 promoter may not be important and the element may simply be a position-dependent enhancer that is located immediately upstream of the Inr consensus. In support of this possibility, mutation of nucleotides at −2 and −3, within the Inr consensus, did not inhibit promoter activity (data not shown).

We thank Dr. P. Kincade for M/K-1. These studies were supported by National Institutes of Health Grants HL29594 and HL43418 and a Career Investigator Award from the American Lung Association (D.C.D.). M.F.I. was supported by National Institutes of Health Training Grant HL07317.

1. Elices, M. J., Osborn, L., Takada, Y., Crouse, C., Luhosky, S., Hemler, M. E. & Lobb, R. R. (1990) *Cell* **60**, 577–584.
2. Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi, R. G. & Lobb, R. (1989) *Cell* **59**, 1203–1211.
3. Rice, G. E., Munro, J. M., Corless, C. & Bevilacqua, M. P. (1991) *Am. J. Pathol.* **138**, 385–393.
4. Hemler, M. E., Huang, C. & Schwarz, L. (1987) *J. Biol. Chem.* **262**, 3300–3309.
5. Erle, D. J., Ruegg, C., Sheppard, D. & Pytela, R. (1991) *J. Biol. Chem.* **266**, 11009–11016.
6. Freedman, A. S., Munro, M., Rice, G. E., Bevilacqua, M. P., Morimoto, C., McIntyre, B. W., Rhynart, K., Pober, J. S. & Nasdler, L. M. (1990) *Science* **249**, 1030–1033.
7. Miyake, K., Weissman, I. L., Greenberger, J. S. & Kincade, P. W. (1991) *J. Exp. Med.* **173**, 599–607.
8. Scheeren, R. A., Koopman, G., Van der Baan, S., Meijer, C. J. & Pals, S. T. (1991) *Eur. J. Immunol.* **21**, 1101–1105.
9. Rice, G. E., Munro, J. M. & Bevilacqua, M. P. (1990) *J. Exp. Med.* **171**, 1369–1374.
10. Carlos, T., Kovach, N., Schwartz, B., Rosa, M., Newman, B., Wayner, E., Benjamin, C., Osborn, L., Lobb, R. & Harlan, J. (1991) *Blood* **77**, 2266–2271.
11. Shimizu, Y., Newman, W., Gopal, T. V., Horgan, K. J., Graber, N., Beall, L. D., van Seventer, G. A. & Shaw, S. (1991) *J. Cell Biol.* **113**, 1203–1212.
12. Ruegg, C., Postigo, A. A., Sikorski, E. E., Butcher, E. C., Pytela, R. & Erle, D. J. (1992) *J. Cell Biol.* **117**, 179–189.
13. Rosen, G. D., Sanes, J. R., LaChance, R., Cunningham, J. M., Roman, J. & Dean, D. C. (1992) *Cell* **69**, 1107–1119.
14. Iademarco, M. F., McQuillan, J. J., Rosen, G. D. & Dean, D. C. (1992) *J. Biol. Chem.* **267**, 16323–16329.
15. Yaffe, D. (1968) *Proc. Natl. Acad. Sci. USA* **61**, 477–483.
16. Birkenmeier, T. M., McQuillan, J. J., Boedeker, E. D., Argraves, W. S., Ruoslahti, E. & Dean, D. C. (1991) *J. Biol. Chem.* **266**, 20544–20549.
17. McBurney, M. W. & Rodgers, B. J. (1982) *Dev. Biol.* **89**, 503–508.
18. Rosen, G. D., Birkenmeier, T. M. & Dean, D. C. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4094–4098.
19. Miyake, K., Medina, K., Ishihara, K., Kimoto, M., Auerbach, R. & Kincade, P. W. (1991) *J. Cell Biol.* **114**, 557–565.
20. Weintraub, S. J. & Dean, D. C. (1992) *Mol. Cell. Biol.* **12**, 512–517.
21. Kemler, I., Bucher, E., Seipel, K., Muller-Immergluck, M. M. & Schaffner, W. (1991) *Nucleic Acids Res.* **19**, 237–242.
22. Dean, D. C., McQuillan, J. J. & Weintraub, S. J. (1990) *J. Biol. Chem.* **265**, 3522–3527.
23. Haggarty, A., Rina, C., Paterno, G., Cohen, L., Hiscott, J. & Skup, D. (1991) *Cell Growth Dev.* **2**, 503–510.
24. Dean, D. C., Bowlus, C. L. & Bourgeois, S. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1876–1880.
25. Tanaka, M. & Herr, W. (1990) *Cell* **60**, 375–386.
26. Dent, C. L., Lillycrop, K. A., Estridge, J. K., Thomas, N. S. & Latchman, D. S. (1991) *Mol. Cell. Biol.* **11**, 3925–3930.
27. Smale, S. Y. & Baltimore, D. (1989) *Cell* **57**, 103–113.

1: DNA Cell Biol 2000 Aug;19(8):507-14

Cooperative binding of TEF-1 to repeated GGAATG-related consensus elements with restricted spatial separation and orientation.

Jiang SW, Desai D, Khan S, Eberhardt NL.

Endocrine Research Unit, Department of Medicine, Mayo Clinic, Rochester, Minnesota 55905, USA.

The human transcriptional enhancer factor (TEF) family includes TEF-1, TEF-3, TEF-4, and TEF-5. The TEFs share a highly conserved 68-amino acid TEA/ATTS DNA-binding domain, which binds to SV40 GT-IIC (GGAATG), SphI (AGTATG), SphII (AGCATG), and muscle-specific M-CAT (GGTATG) enhansons. We determined the optimal DNA-binding consensus sequence for TEF-1. Using a purified GST-TEF-1 fusion protein and a random pool of synthetic oligonucleotides, 31 independent clones were obtained after six rounds of binding site selection. DNA sequences analysis revealed that 16 clones contained direct repeats with a 3-bp spacer (DR3), and 15 clones contained a single binding site. The predominate consensus half-site was GGAATG (67%), and the other elements were of the form G(A)GA(T/C)ATG. The TEF-1 bound to the DR3 as a dimer in a cooperative manner. Cooperative binding was dependent on the spacing and orientation of the half-sites and was inhibited by deoxycholate treatment, providing evidence that protein-protein interactions were involved. The data suggest that TEF dimerization is important for its ability to modulate gene transcription.

PMID: 10975468 [PubMed - indexed for MEDLINE]